Co-localization of β1,6-Branched Oligosaccharides and Coarse Melanin in Macrophage-Melanoma Fusion Hybrids and Human Melanoma Cells In Vivo

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YALE UNIVERSITY

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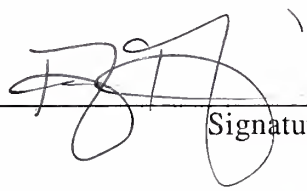

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A Thesis Submitted to the

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in Partial Fulfillment of the Requirements for the

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by

Reena N. Rupani

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ABSTRACT

Rupani R.N., Pawelek J.M. Co-localization of β1,6-Branched Oligosaccharides and Coarse Melanin in Macrophage-Melanoma Fusion Hybrids and Human Melanoma Cells *In Vitro*. Department of Dermatology, Yale University School of Medicine, New Haven, CT.

Fusion hybrids between normal macrophages and Cloudman S91 melanoma cells were shown in previous studies to have increased metastatic potential, along with high expression of β1,6-N-acetylglucosaminyltransferase V (GnT-V) and β1,6-branched oligosaccharides. Curiously, hybrids, but not parental melanoma cells, also produced 'coarse melanin'-- autophagic vesicles with multiple melanosomes. Since β1,6-branched oligosaccharides were known to be associated with metastasis, and coarse melanin had been described in invasive human melanomas, the purpose of this study was to look for potential relationships between the two. Using lectin/immunohistochemistry, we analyzed cell lines producing coarse melanin for β1,6-branched oligosaccharides; gp100/pmel-17, a melanosomal structural component; and CD63 a late endosome/lysosome component highly associated with melanoma and certain other human cancers. Cell lines were hybrid 94-H48, a highly metasatatic, macrophage-melanoma artificial fusion hybrid; 6^{neo} mouse melanoma cells, the weakly metastatic, parental fusion partner; and SKMel-23, a human melanoma cell line derived from a metastasis. Coarse melanin granules were prominent in both hybrids and SKmel-23 cells, and co-localized with stains for β1,6-branched oligosaccharides, gp100/pmel 17, and CD63. Co-expression of β1,6-branched oligosaccharides and coarse melanin was recently shown to be a common and pervasive characteristic in archival specimens of human melanomas (most prominently in metastases), but this is the first report of this phenotype being expressed *in vitro*. Such expression in both human melanoma cells and experimental macrophage-melanoma fusion hybrids provides new biological systems for more detailed analyses of its genesis and regulation at the molecular genetic level.

ACKNOWLEDGEMENTS

At the risk of sounding kitsch-y, I am taking this page as an opportunity to speak in my own plain dialect, devoid of any proper scientific lingo, and thus reflect on what this experience has meant to me. I am a girl from Kansas whose childhood memories are suffused with music/dance and performance art, whose college years were dedicated to the Dismal Science (here I refer affectionately to a BA in economics), whose brief post-grad professional life was spent as a Strategy Consultant...and now, with spring 2004 on its glorious verge, who's gonna be a doctah. It feels great! I have enjoyed my time at Yale immensely, and the research herein described plus all of the people involved have been such an indelible part of my four years.

So with that, we are now mid-page, and what I have left to say is 'thank you'— first and foremost to Dr. John Pawelek, who is not only my P.I., but who has also become both friend and family. You embody my ideals of what an entrepreneur should be, in every sense of the word. A heartfelt thank you also to Stefano Sodi and Ashok Chakraborty for all of your wonderful teaching, and for allowing me to be "Dr. Rupani" in lab; to my buddy Vincent Klump in dermpath, who frankly just runs the show; to James Platt, who anyone that owns a computer should definitely have on his/her side; to Tamara Handerson, an excellent dermatopathologist and conference companion; to the Office of Education, for warm support and generous funding; and of course to my family and friends, for being who you are.

One vocal artist musically proclaimed in her recent Grammy performance that she is 'dangerously in love,' and I must borrow some of this sentiment and phrasing to say that I am 'dangerously in debt' to all of you named above; thank God, however, that this sort of 'debt' has no fiscal implications (I recently consolidated all of my loans)! This thesis is my only collateral.

....Alright, that's enough for now I suppose.

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INTRODUCTION

A detailed discussion of melanoma, which is principally (but not exclusively) a cancer of epidermal melanocytes, first requires a succinct review of the skin itself. As the body's largest organ, the skin serves a variety of functions including, fundamentally, the maintenance of a constant internal environment. In order to accomplish this, the skin functions in photoprotection, thermoregulation, cutaneous circulation, barrier formation, and immunologic protection (1).

The outer layers of the skin comprise the epidermis, which is subdivided from top to bottom into the stratum corneum, stratum granulosum, stratum spinulosum, and stratum basale.

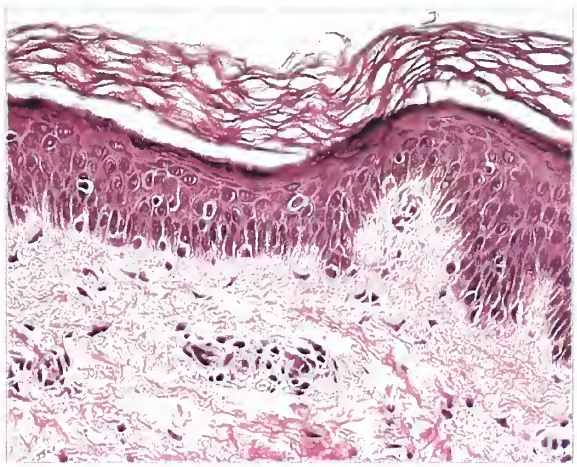


Figure 1. Human epidermis/dermis. The five layers of the epidermis are visible from top to bottom.

The major cellular components of the epidermis are keratinocytes, melanocytes, and Langerhans cells; melanocytes are embryologically-derived from the neural crest, and with their capacity to elaborate the radiation-absorbing pigment melanin, play a primary role in protecting the skin and body from ultraviolet radiation damage. Melanin is

packaged into melanosomes, which are subsequently transferred through melanocyte dendritic processes into neighboring keratinocytes, where they then reside in a perinuclear location (1).

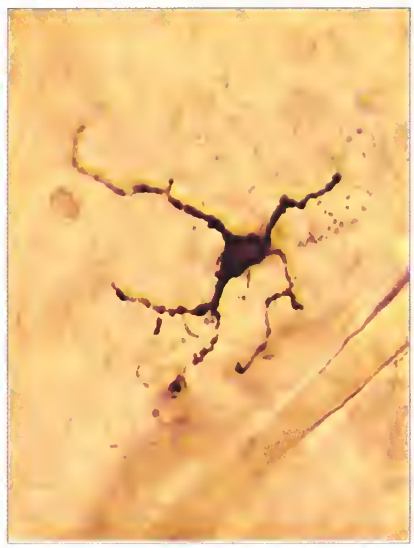


Figure 2. Normal human melanocyte stained for tyrosinase.

Finally, beneath the epidermis lies the dermis, a mucopolysaccharide gel bound by a collagen and elastin-containing fibrous matrix, which is vascularized to provide both nutritional and structural support to the skin (1).

Given this brief structural and functional outline of normal epidermis and dermis, we turn to the example of disordered epidermal cellular function which is the particular focus of this study: melanoma, a malignant tumor arising from melanocytes. The incidence of melanoma and its associated mortality rates are increasing, particularly amongst younger individuals (1). Diagnosis includes attention to early sunburns and intense intermittent exposure to sun in individuals with types I or II skin (although melanoma in darker-skinned individuals can occur on non-sun-exposed areas such as

acral sites). Clinical features are characterized by the ABCD mnemonic for evaluation of any suspicious lesions (asymmetry, border, color variation, diameter), and studies have shown that melanomas present more typically on the trunk in males and on the lower extremities in females (1). Histology reveals cellular atypia, mitoses, and invasion of the epidermis (2), and tumor thickness remains the most important prognostic indicator in primary melanoma (i.e., Breslow thickness scale). Melanomas can be extremely aggressive if not discovered early enough; metastases can occur in various organs including the lungs, liver, and central nervous system, with homing behavior often following the neural crest pattern of embryologic origin, resulting also in cutaneous metastases.

An important question is how or why, in fact, such metastatic spread occurs. Key components of metastasis include an imbalance in regulation of adhesion, motility, and proteolysis, but the origins of cells with a metastatic phenotype are as yet unknown (3). The metastatic transformation of a primary melanoma is likely a multimodal process: "There are of course numerous examples correlating cancer with genetic changes, and in primary tumors many gene mutations have been identified that lead to cellular immortalization. But fewer gene mutations have been identified that correlate with metastasis, and indeed the strongest correlation for genetic changes in metastasis is aneuploidy as opposed to direct gene mutation." (4). In thinking of metastasis as an evolutionary process (i.e., a process whereby acquired genetic variability allows the selection of more aggressive cell sublines [5]), there arises the question of whether there is a role for hybridization in the generation of cellular aneuploidy—meaning, a genetic fusion event between a phagocyte and a melanoma cell (e.g., during the abortive

digestion of apoptotic tumor cells by phagocytes), thereby yielding a hybrid which demonstrates features of both parental lines including myeloid-type mobility and the deregulated growth of cancer cells.

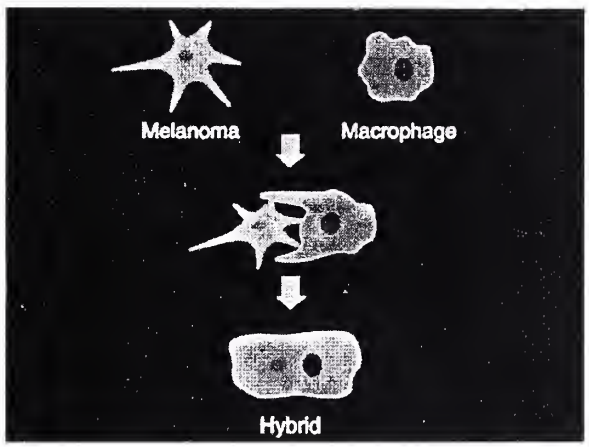


Figure 3. Macrophage x melanoma fusion hybrids (6).

Thus, as the genes for mitochondria and chloroplasts were acquired via endosymbiosis between pro-eukaryotes and prokaryotic cells, such is the logic for the theory of hybrid formation between myeloid and primary tumor cells (4).

The idea of hybridization as a source of aneuploidy in cancer dates back to the research of Theodor and Marcella Boveri in the late 1800s: Their work with sea urchin eggs, which were experimentally fertilized with two different sets of spermatozoa, resulted in abnormal mitoses and led to the speculation that chromosomal imbalance might also play a role in the deregulated growth of cancer cells (7). These findings prompted Aichel in the early 20th century to extend the notion of chromosomal excess to human cancers, specifically the idea that hybridization between leukocytes and cancer cells might lead to genetic pooling/chromosomal imbalance, and therefore abnormal mitoses (8-9). Finally, Mekler in 1971 formulated the concept that hybridization between

immortalized tumor cells and leukocytes might confer properties of motility (i.e., that which is necessary for metastasis) to cells of the primary tumor (10). Indeed, today it is well-documented in animal models that tumor hybrids occur spontaneously (11-13). Also, leukocyte x lymphoma and leukocyte x melanoma fusion hybrids demonstrate increased moility *in vitro* and metastatic potential *in vivo* (11-13).

With specific regard to melanoma there have been many studies, including those by Munzarova et al, Pernick et al, and others (14-16), which noted not only the expression of macrophage-like traits by metastatic melanoma (such as invasive behavior, chemotactic migration with reversible adhesive contacts, accumulation in draining lymphoid tissue, extravasation and release into circulation [17]) but also the presence of known histiocytic markers such as Mac-1 and CD68 on metastatic melanoma cells, as evidenced by immunoreactivity (16, 18-19). Both artificial and naturally-occuring tumor x host hybrids in mouse models were previously studied and noted to have enhanced metastatic capabilities (20-31).

Thus, it was in the context of this historical and experimental background that the theory of macrophage-melanoma cell hybridization was tested in this thesis. This study in particular centered around a known phenomenon in highly metastatic cell lines and solid tumors (including breast, colon and others [32]), which is an increased level of β1,6-branched N-glycosylation of integral membrane proteins. Such proteins include lysosome-associated membrane proteins (LAMPs) 1&2, which are over-expressed in malignant cells and likely serve to stabilize lysosomes for transport to the cell membrane, with subsequent extracellular release of lysosomal enzymes for degradation of surrounding tissue (6); β-integrins, essential for cell adhesion to fibronectin; cadherins,

involved in Calcium-mediated cell-cell adhesion properties; matriptase, which (among other functions) activates the HGF/cmet motility pathways; and other proteins which are as yet uncharacterized. This form of glycosylation is catalyzed by the myeloid-type enzyme GnT-V, or β1,6-N-acetyl-glucosaminyltransferase-V.

β1,6-branched glycosylation is employed in motility by both myeloid cells and cancer cells, conferring changes in substrate adhesion, loss of contact inhibition, and activation of motility-promoting integrins such as $\alpha 5\beta_1$ (6, 32-34). β1,6-branching is catalyzed by the trans-Golgi enzyme GnT-V (E.C.2.4.1.155) and initiates the addition of polylactosamine antennae to the mannose-rich core of N-glycans.

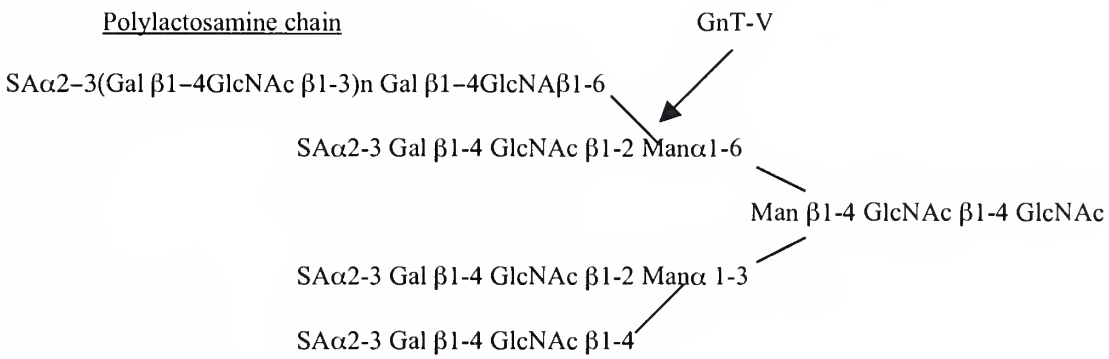


Figure 4. N-acetylglucosaminyltransferase V (GnT-V) involved in β 1,6 branch formation and polylactosamine chain synthesis. Man - mannose, GlcNAc - N-acetylglucosamine, Gal - galactose, SA - sialic acid.

As principal carriers of N-acetylpolylactosamine antennae, LAMPs-1 and -2 are the most densely N-glycosylated of cellular proteins; synthesis of these antennae is catalyzed by GnT-V in the trans-golgi apparatus, where β1,6 branches are formed on the trimannosyl terminus of Asn-linked oligosaccharides (35). This in turn facilitates the enzymatic addition of β1,6-GlcNAc-linked poly-N-acetyllactoseaminyl chains by UDPGlcNAc-N-acetyllactoseaminide β1,3-N-acetylglucosaminyl transferase (36). These

polylactosamines are carriers of Lewis^x and Lewis^a antigens, used in selectin binding during intravasation and systemic migration, and associated with poor patient prognosis in human cancers (6, 32-34). Thus, in summary, the enzyme GnT-V regulates the polylactoseamine contents of N-linked oligosaccharides with its activity serving as an indication of β1,6-branching, and elevated GnT-V activity is characteristic of both myeloid cells and metastatic melanoma cells (35).

In our lab's previous studies of artificial fusion hybrids between normal macrophages and a weakly metastatic mouse melanoma cell line, approximately half of the hybrids demonstrated GnT-V expression and β1,6-branched glycosylation similar to that of the parental macrophages and three to four times that of the parental melanoma cells (35). This correlated with dramatic phenotypic changes that, at least in some cases, appeared to be due to altered N-glycosylation. For example, hybrid clones with elevated GnT-V showed enhanced chemotactic motility *in vitro* that was suppressed by N-glycosylation inhibitors such as swainsonine and castanospermine (17, 37). Hybrids with elevated β1,6-branched glycosylation also tended to be highly pigmented, as opposed to the nonpigmented parental melanoma cells. Enhanced pigmentation correlated with elevated N-linked glycosylation of melanosomal proteins tyrosinase (the rate-limiting enzyme in melanogenesis), TYRP-1 (tyrosinase-related protein 1), dopachrome tautomerase (TYRP-2), and LAMP-1, and was again suppressed by N-glycosylation inhibitors and glycosidases (38-39). Hybrids also acquired enhanced responsiveness to inducers of intracellular cyclic AMP-- MSH (melanocyte-stimulating hormone,

melanocortin-I) and IBMX (isobutylmethylxanthine, cholera toxin)-- with increased pigmentation, motility, and dendricity (17, 35, 37-39).

A curious finding was that melanosomes in artificial melanoma-macrophage (both mouse and human) hybrids were often packaged together in heterogeneous 'coarse melanin' vesicles, rather than existing free in the cytoplasm as in normal melanocytes and parental melanoma cells. Coarse melanin was first described in relation to animal-type melanoma in 1832, and then in humans in 1925 (40). It was described by Clark et al as a major melanosomal abnormality primarily associated with cells in vertical growth phase melanomas, consisting of numerous granular organelles, 250-500nm in width with melanin deposited in a coarsely granular fashion, commonly seen in superficial spreading melanoma, in nodular melanoma, and in metastatic melanoma (41).

Similarly, recent studies from our laboratory of human archival melanoma and 21 other cancers universally revealed for the first time the production of 'coarse' autophagosomal vesicles, apparently a shared phenotype associated with elevated GnT-V activity (32). This phenotype was an independent predictor of metastasis and poor survival in melanoma, breast, and colon carcinomas. In melanomas, the autophagosomes were often laden with coarse melanin, accounting for the well-known hypermelanotic regions of primary tumors used in clinical diagnosis. The association between this phenotype and elevated GnT-V activity was established via immunoperoxidase staining for a particular marker of β1,6-branched structures known as LPHA, as well as by staining with antibodies against HMB45 and CD63.

LPHA, or leukocyte-phyto-hemagglutinin, is a lectin derived from the plant *phaseolus vulgaris* which binds to and thus identifies β1,6-branched structures. HMB45

is an antibody to human melanosomal membrane protein gp100 (pmel17 is the mouse homolog, used herein). CD63 is a glycoprotein that was first discovered as a melanoma-associated antigen (42-44) but has since been found in a number of other neoplasms and normal cells (32, 45-46). CD63 belongs to the transmembrane 4 superfamily, or tetraspanins, a large family of membrane proteins associated with cell proliferation, cell migration, and tumor cell invasion (47-49), in part through affecting integrin signaling and differential adhesion (50-54). CD63 is usually found in association with LAMPs 1 and 2, both major substrates for GNT-V, and it would thus be expected to co-localize to structures containing β1,6-branched N-glycans.

There have been previous studies documenting vesicle formation in various other models, with a postulated link to GnT-V activity. Fukuda et al, for example, discussed a case of a pigmented renal cell carcinoma which demonstrated collections of "abnormal lysosomal granules" (55). It was also of considerable interest to us that Hariri et al (56) reported formation of autophagy-dependent "multilamellar vesicles" following transfection of GnT-V into non-neoplastic mink alveolar type II lung cells. The vesicles were decorated with β1,6-branched N-glycans and contained both LAMP-2 and CD63 (56).

PURPOSE

Thus, in the setting of historical knowledge of GnT-V activity and aberrant glycosylation in neoplastic cells, and previous findings by our lab of increased GnT-V levels and a coarse vesicular phenotype in human melanomas and other solid tumors, the purpose of this study was to confirm said findings in an *in vitro* model via histochemical analyses of known GnT-V substrates/markers in an artificial mouse macrophage-melanoma hybrid cell line and a human melanoma cell line. It was hypothesized that coarse melanin vesicles in macrophage-melanoma fusion hybrids might also be related to GnT-V expression. As such, I show below that melanized autophagosomal vesicles in experimental macrophage-melanoma fusion hybrids were indeed rich in β1,6-branched N-glycans, and that such structures were also a feature of human melanoma cell lines *in vitro*. The biological significance of this phenotype is not understood; however, the strong relationship to metastasis makes it of obvious interest. The cultured cell lines provide new biological systems for analysis of GnT-V regulation and *N*-linked glycosylation at the molecular genetic level.

MATERIALS AND METHODS

Two cell lines utilized in this study (S91-6^{neo} and hybrid 94-H48) were previously generated in our lab by Dr. John Pawelek and Michael Rachkovsky. Cells were prepared for electron microscopy by Dr. John Pawelek. Experiments and analysis conducted by myself included the following: Cell culture and maintenance, fixation (protocol kindly provided by Dr. Ruth Halaban, Ph.D., Yale University Department of Dermatology), bleaching (protocol kindly provided by Vincent Klump, Yale University Department of Dermatopathology) and immunoperoxidase staining; additionally, under the guidance of Dr. John Pawelek, I photographed cells under both the electron and light microscopes; I prepared cells for FACS analysis under the supervision of Dr. Ashok Chakraborty, as well as carried out the experiments and ensuing computer data analyses; transfection experiments (see Discussion) were performed by Dr. Ashok Chakraborty and cells were analyzed by myself under the light microscope; finally, I performed all cell counts and statistical analyses.

Cell lines: As stated above, two of the cell lines here studied, namely S91-6^{neo} and hybrid 94-H48, were previously generated in our lab by other researchers. Methods are here paraphrased (17): Melanoma cells and macrophages were maintained in HAM's F10 or Dulbeco's MEM media with the addition of 10% fetal bovine serum (mouse cells) or 15% human serum (human macrophages). Cells were maintained in a gassed and humidified incubator (5% CO2 in air, 37°C). The cell line used as a control partner for fusion with macrophages was identified as Cloudman-S91 (PS-1-HGPRT-1), and generated as follows: The S91 tumor originated as a malignant melanoma in the tail base

of a DBA female mouse, and after subsequent transplantation to another mouse tail, showed a striking amount of highly-pigmented pulmonary metastases (57). Via subsequent transplantations into different DBA mice, this tumor was maintained through successive generations. Clone M3 was adapted to culture from a transplanted S91 tumor and deposited with the American Type Culture Collection (58), then manipulated in our laboratory. PS1-HGPRT-1 was again isolated by resistance to azaguanine and thioguanine, then further selected for resistance to neomycin (which typically destroys macrophages). Similarly, spontaneous hybrids within this clone were isolated by resistance to hypoxanthine, aminopterin, thymidine (HAT, which typically destroys S91 cells) and/or neomycin, as hybrids are resistant to both neomycin and HAT.

Peritoneal macrophages were obtained from DBA/2J mice four days after injection with 3ml of thioglycollate broth (Difco) and immediately prepared for fusion experiments. Human blood monocytes were donated by a member of the lab, isolated by standard Ficoll density centrifugation techniques, and kept in monolayer culture for 72 hours before use in fusion experiments (17).

Creation of the hybrid cell line was accomplished previously by polyethylene glycol fusion (PEG), and is here paraphrased (17): PS1-HGPRT-1 neomycin –resistant Cloudman-S91 cells were mixed with macrophages (separately mouse and human) at respective ratios (tumor/macrophage) between 1:4 to 1:10. Polyethylene glycol (PEG 1450, Sigma Chemicals) was dissolved by warming and mixed in a 50:50 volume ratio with Ham's F10 serum-free nutrient medium. Mixed PEG/F10 (0.8ml) was added to centrifuged cell pellets over a period of 1 minute at 37° C, with gentle mixing. The suspension was maintained at 37° C and continuously mixed for an additional minute;

this procedure was followed by the addition of 1ml of Ham's F10 over another minute, and then 20ml of Ham's F10 over 5 minutes. The mixture was finally centrifuged, supernatant withdrawn, and cells seeded into 25 cm^2 Corning tissue culture flasks (concentration 1.5 x 10^5 cells/flask). These flasks were maintained in a gassed and humidified incubator at 37° C for 48 hours, after which time the initial medium was replaced with medium containing HAT (concentrations= hypoxanthine 1 x 10^{-4}, aminopterin 4 x 10^{-7}, thymidine 1.6 x 10^{-5}) +/- neomycin, depending on the experiment. Hybrid cells, then, were identified as those resistant to both HAT and neomycin (17). Only mouse/mouse hybrid cells were ultimately used in this study.

Pigmented human metastatic melanoma cell lines SKmel-23 (clone C22), SKmel-30, and SKmel-188 (59) were kindly provided by Dr. Alan Houghton, Sloane-Kettering Memorial Institute for Cancer Research.

Cell culture: Cells (5 x 10^4), in 0.2ml DMEM/10% fetal bovine serum with penicillin and streptomycin were plated onto Snowcoat sterile glass slides. Medium was changed the following day, and half of the cultures were supplemented with MSH (2 x 10^{-7}M) and 3-methyl isobutylxanthine (10^{-4}M). Cultures were incubated in a gassed (5%CO_2/95% air), humidified incubator at 37°C, reaching 60-75% confluence (by visual approximation) by 3 days when they were processed for lectin/immunohistochemistry. For fixation, cultures were gently rinsed in phosphate-buffered saline, immersed in methanol (100%, 1 min), and then air-dried.

Coarse melanin co-localization. Coarse melanin was identified and photographed in unstained cells using an Olympus AH2 light microscope/camera adapted with Spot™ software, and microscope coordinates of the region were recorded. The sections were bleached (below), then lectin-, or immunostained via standard horseradish peroxidase techniques, counter-stained with hematoxylin, and the same regions were again located and rephotographed.

Bleaching: To decolorize melanin, slides were immersed in tap water for 24 hours to dissolve the water-based mounting medium (Clearmount, Zymed Laboratories Inc.) and passively remove cover slips. The cells were then bleached by immersion in KMnO4 (0.25%, 4 min.); rinsed in distilled water; immersed in oxalic acid solution (1%, 1 min.); washed in gently-running tap water (2 min); and rinsed twice in phosphate-buffered saline to restore pH. The slides were monitored under the light microscope to ensure that bleaching was complete.

Lectin- and Immunohistochemistry: Slides were processed and stained by the Dako™ immunoperoxidase technique: The slides were incubated in peroxidase block for 5 minutes and rinsed in phosphate-buffered saline to restore pH. Biotinylated primary antibodies (described below) were applied, and slides were incubated for a period of 15 minutes before rinsing with PBS. This was followed by a 15 minute incubation in streptavidin-peroxidase and another PBS rinse, and the procedure was completed with a 5-minute incubation in prepared DAB+substrate-chromagen solution and tap water rinse.

After immunoperoxidase staining, each slide was counterstained using filtered Gil III Hematoxylin (10 second rinse), a 1 second immersion in Ammonium Hydroxide (1%), and a running tap water rinse. The slides were then dehydrated in 100% ethanol and mounted with coverslips using a Xylene-based mounting medium (Cytoseal 60 low viscosity, Richard Allan Scientific).

Antibodies and lectins were as follows: HMB45 (anti-human melanosomal component gp100; prediluted by company, 1:50 dilution) was from Dako, Inc., Carpinteria, CA. Anti-pmel-17 (mouse homologue of human gp100, 1:50 dilution) was kindly provided by Dr. Vincent Hearing, US National Cancer Institute. Anti-CD63 (MAbME491; lysosome/late endosome component; 1:20 dilution) was a kind gift of Dr. M. Herlyn, Wistar Institute, Phila. PA. Biotinylated. LPHA (1:50 dilution), for detection of β1,6-branched N-glycans, was from Vector Laboratories, Burlingame, CA.

Electron Microscopy. Fixation/preparation was conducted by other researchers in our lab. Cells were fixed in half-strength Karnovsky's fixative for 6 hrs at 4°C; washed in cacodylate buffer overnight; post-fixed with 1% OsO4 and 1.5% potassium ferrocyanide in cacodylate buffer for 2 hrs; and embedded in Spurr's resin. Ultrathin sections were stained with uranyl acetate and lead citrate, and photographed by this author through a Zeiss 109 electron microscope.

FACS Analysis. Analysis was carried out for LPHA only, in 6^{neo} and hybrid 48 cells (no human melanoma cell analysis). LPHA binding was facilitated by incubating cells with

FITC-conjugated LPHA (10μg/ml; Vector, Burlingame, CA) in phosphate-buffered saline with 0.1% bovine serum albumin and 0.05% sodium azide (to prevent fungal contamination). After 3-4 washings with PBS, cells were fixed with 1% paraformaldehyde. Flow cytometry was carried out using a FACS Vantage flow cytometer (Becton Dickinson). Predominantly cell surface expression of LPHA target ligands was measured, as the antibody and lectin incubations were performed with intact cells on ice.

RESULTS

Quantitation of pmel-17/gp100 and β1,6-branched oligosaccharides. The three cell lines, with and without MSH/IBMX treatment, were scored as to the percentage of cells in the population staining for LPHA and anti-pmel-17/gp100 (Figure 5).

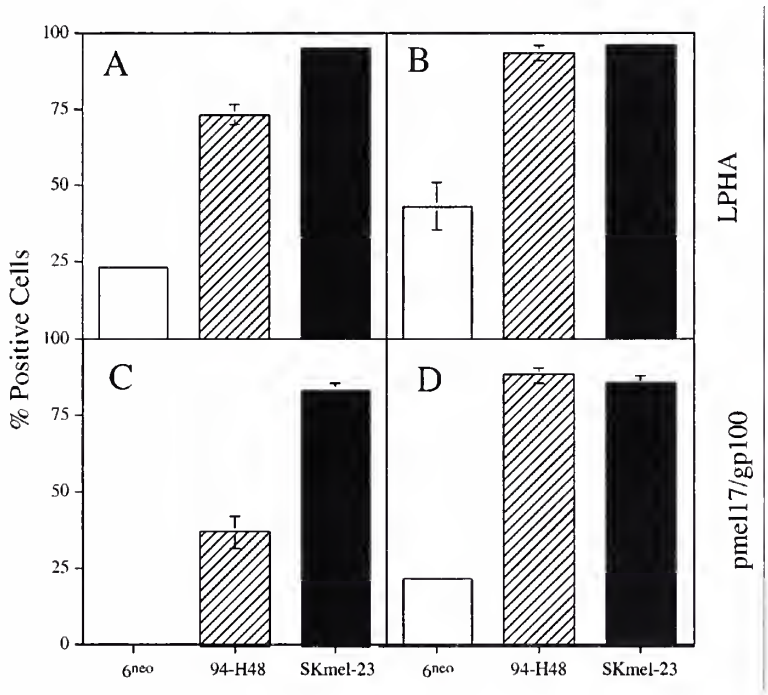


Figure 5. Relative expression of pmel-17/gp100 and β1,6-branched oligosaccharides in cell lines 6^{neo}, hybrid 94-H48, and SKmel-23, cultured in the absence and presence of MSH/IBMX (Methods). Following staining, the three lines were scored under a light microscope as to the percentage of cells positive for LPHA or anti-pmel-17 (mouse)/gp100 (human). Results represent mean +/- S.D. for three separate counts of 100-200 cells each. A. LPHA, control; B. LPHA, MSH/IBMX; C. anti-pmel-17/ gp100, control; D. anti-pmel-17/ gp100, MSH/IBMX.

In the absence of MSH/IBMX, hybrid 94-H48 and SKmel-23 cells each showed a higher percentage of LPHA- and HMB45-positive cells compared to parental 6^{neo} melanoma cells, and for hybrid cells this was increased further following exposure to MSH/IBMX. (*P* values were computed by Student's t-Test using the Statview™ program, and for these differences were all less than *P*=.0060.) In the absence of MSH/IBMX, SKmel-23 cells

showed even higher numbers of positive cells than did hybrid 94-H48, with >90% of the population positive for LPHA and >80% positive for HMB45. Exposure of SKmel-23 cells to MSH/IBMX did not further increase this already high percentage of positive cells, although it did promote an increase in dendrites (not shown).

On a 'per cell' basis, the staining intensity in hybrid 94-H48 and SKmel-23 cells was estimated (by visual approximation) as about 4-5 times greater than that seen in parental 6^{neo} cells, consistent with flow cytometry data conducted previously by other members of our lab (35) (Figure 6).

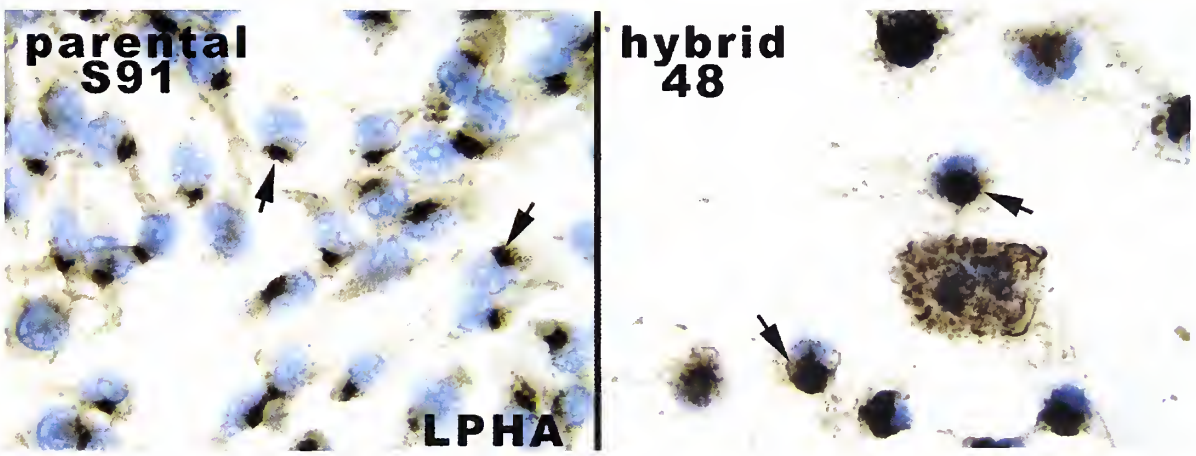


Figure 6. Comparison of parental 6^{neo} melanoma cells and hybrid 94-H48 following staining with LPHA. Left: 6^{neo}, arrows denote LPHA staining. Right: 94-H48, arrows denote LPHA staining. Cells were treated with MSH/IBMX.

Thus, from both 'population percentage' data and 'per cell' estimates, hybrid 94-H48 and human melanoma SKmel-23 each showed significantly higher expression of pmel-17/gp100 and β1,6-branched oligosaccharides compared to parental 6^{neo} melanoma cells.

Coarse melanin in hybrid 94-H48. H&E staining revealed little to no melanin in the

parental 6^{neo} cells, but most of the hybrid cells were heavily pigmented with coarse, dark melanin throughout the cytoplasm (Figure 7).

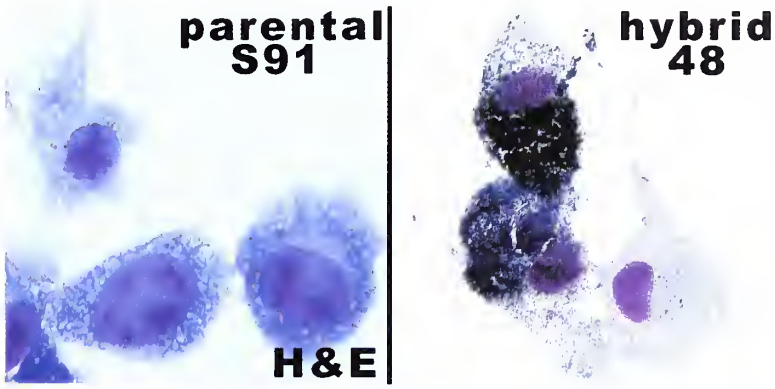


Figure 7. Comparison of parental 6neo melanoma cells and hybrid 94-H48 following staining with H&E. Left: 6^{neo}, H&E stain; lighter perinuclear area denotes Golgi region. Right: hybrid 94-H48, H&E stain; dark, coarse melanin visualized in perinuclear region. Cells were treated with MSH/IBMX.

Electron micrographs revealed that although some melanosomes existed freely in the cytoplasm, the coarse granular appearance of melanin in hybrid 94-H48 was due to melanosome-filled autophagasomes in various stages of maturation (60) (Figure 8).

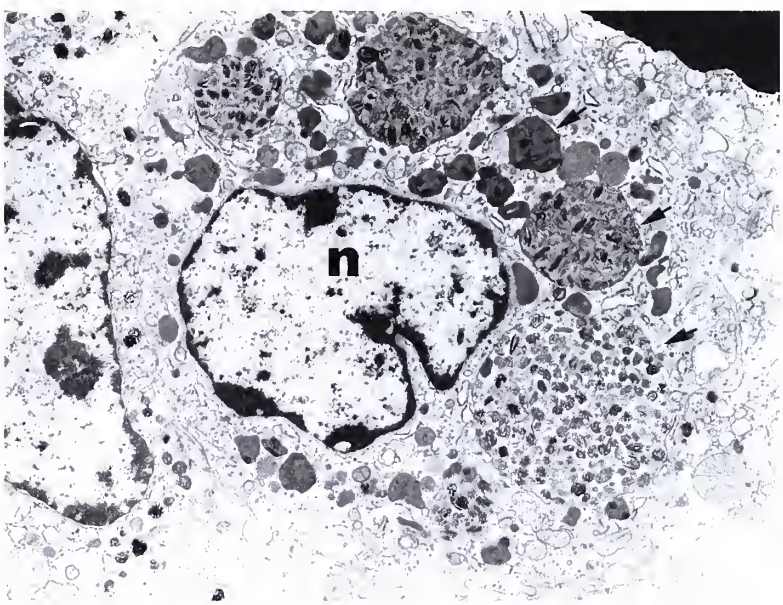


Figure 8. Electron micrograph of macrophage-melanoma hybrid 94-H48 in culture with coarse melanin (melanosome-containing autophagosomes (arrows) in various stages of maturation). (n, nucleus; magnification, 30,000x). Cells were treated with MSH/IBMX.

LPHA staining of 6^{neo} cells revealed an acentric, perinuclear pattern, highlighting the Golgi region (Figure 7 above, left). LPHA staining in hybrid 94-H48 was far more extensive, resembling the melanization pattern and encompassing large portions of the cytoplasm to obscure the nucleus (Figure 7 above, right).

Co-localization studies in hybrid 94-H48 cells, revealed that both LPHA and anti-pmel-17 co-localized to coarse melanin, demonstrating that these structures contained both β1,6-branched oligosaccharides and melanosomal matrix proteins (Figures 9 & 10).

Figures 9 and 10 (below). Mouse macrophage-melanoma hybrid 94-H48. Co-localization of coarse melanin with β1,6-branched oligosaccharides and pmel-17. Left: unstained sections; right: the same sections bleached and stained with LPHA (upper) or anti-pmel-17 (lower). Arrows denote examples of co-localized structures (left vs right panels). Cells were treated with MSH/IBMX.

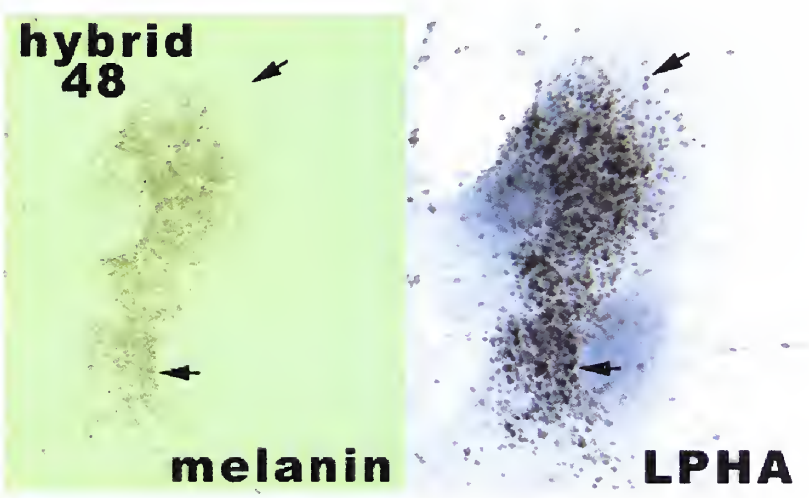


Figure 9

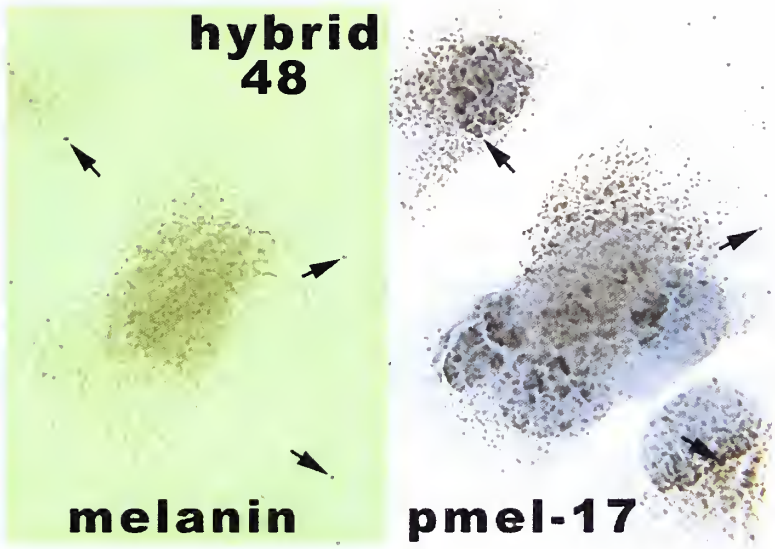


Figure 10

CD63 was not investigated in depth in the mouse cell lines, because the antibodies available were antihuman and did not cross-react well with mouse melanoma cells.

Coarse melanin in human melanoma cell line SKmel-23. SKmel-23 human metastatic melanoma cells exhibited prominent dendrites, often in a bipolar morphology. Melanin granules were seen throughout the cell body and dendrites, and appeared to protrude from the membrane surface (Figure 11).

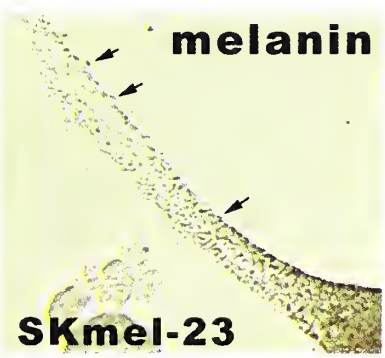


Figure 11. Human SKmel-23 melanoma cell with coarse melanin.

Co-localization studies revealed that the melanin granules stained with LPHA (Figure 12), anti-HMB45 (Figure 13), and anti-CD63 (Figure 13).

Figures 12-14 (below). Human SKmel-23 melanoma cells. Co-localization of coarse melanin with β1,6-branched oligosaccharides, gp100, and CD63. Left: unstained sections; right: the same sections bleached and stained with LPHA (upper), anti-HMB45 (middle), or anti-CD63 (lower). Arrows denote examples co-localized structures (cf left vs right). Cells were treated with MSH/IBMX.

Figure 12

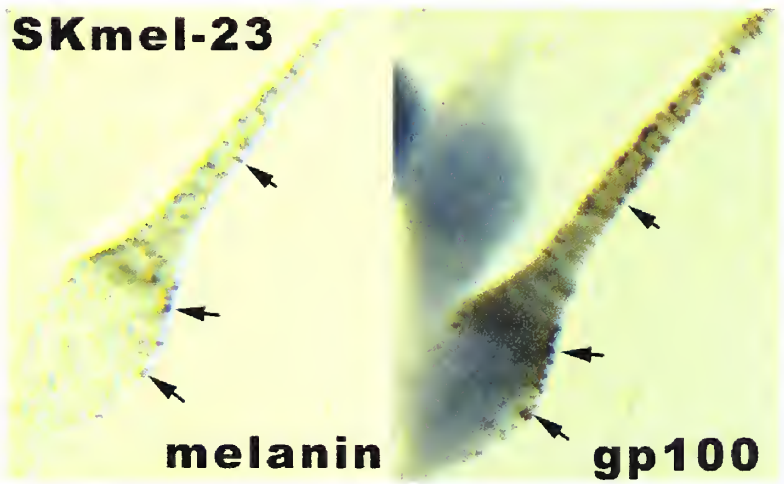


Figure 13

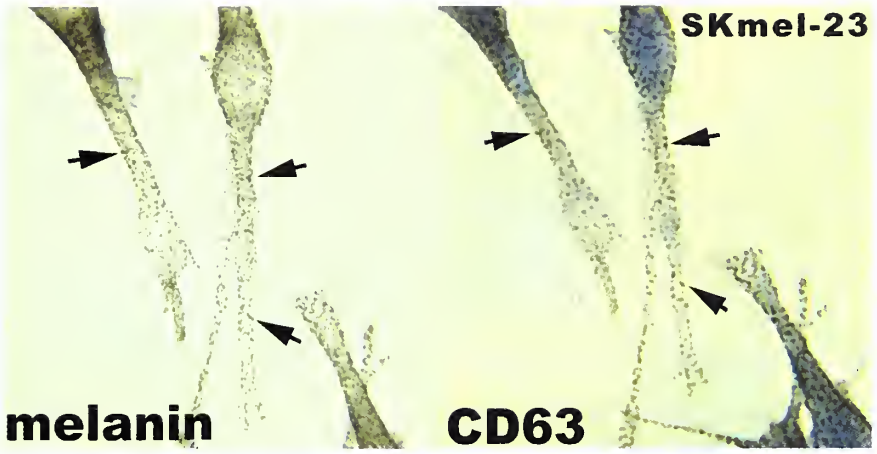


Figure 14

Electron microscopy demonstrated that although some melanosomes were free in the cytoplasm, most were packaged in autophagosomes and multilamellar bodies (Figure 15).

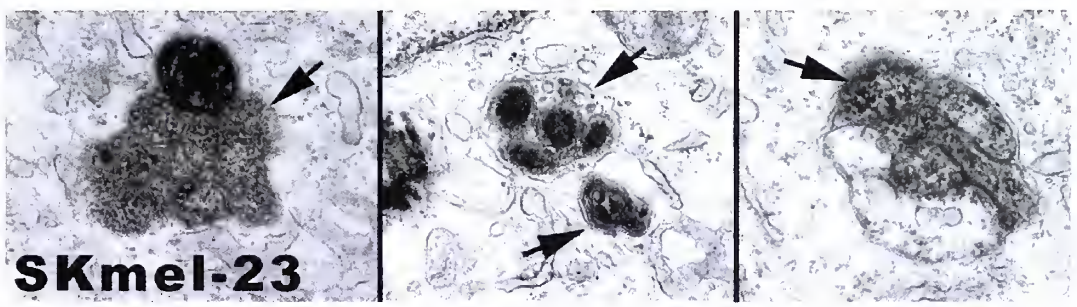


Figure 15: Electron micrographs of human SKmel-23 cells. Each panel is an example of coarse melanin from a separate cell. Cells were treated with MSH/IBMX.

LPHA-positive coarse vesicles were also observed in pigmented human melanoma cell lines SKmel-30 and SKmel-188 (not shown).

FACS Analysis. Analysis of LPHA staining was carried out on 3 separate occasions for both the 6^{neo} and hybrid cell lines, but data was inconsistent/inconclusive overall and therefore not here included. Possible explanations include errors in FACS preparation technique, as well as the use of cells placed in culture at different points in time.

DISCUSSION

In the present studies, a newly-described cancer phenotype in humans, i.e. co-expression of coarse vesicles and β1,6-branched oligosaccharides (32), has been demonstrated *in vitro*. I have shown here that the phenotype was expressed in cultured human melanoma cells and could also be generated through fusion hybridization between normal macrophages and mouse melanoma cells. In macrophage fusion hybrids, the phenotype was enhanced by exposure to MSH in combination with IBMX, a potent stimulus for cyclic AMP production. SKmel-23 cells strongly expressed the phenotype whether or not they were treated with MSH/IBMX.

GnT-V is normally elevated in macrophages and other phagocytes, where β1,6-branched polylactosamines with terminal Lewis^x and Lewis^a antigens are employed for selectin binding during intravasation and systemic migration (61-64). In the macrophage fusion hybrids described here, it is tempting to speculate that GnT-V was expressed as a transcriptionally dominant, developmentally-imprinted myeloid trait. In any case, since an LPHA-positive, vesicular phenotype is a characteristic of normal migratory cells, this might provide clues as to the relation between this phenotype and migration of malignant cells.

It is also notable that human melanoma-associated melanophages are laden with LPHA-decorated, melanin-containing vesicles, thought to be phagolysosomes from phagocytosed melanoma cells (32). In mammalian cells, the process of macroautophagy is a known mechanism for degradation of intracellular macromolecules and organelles, thus playing a prominent role in controlling cell growth and protein metabolism (32). Indeed, by electron microscopy of cultured human Skmel 23 melanoma cells and

previous ultrastructural studies of human melanoma, results indicated that coarse melanin in melanoma is autophagosomal (4, 35; 65-69). Interestingly, GnT-V expression has also been shown to induce multilamellar bodies in mink type II alveolar lung cells through autophagy pathways, raising the possibility that the coarse vesicles observed herein might have been generated through the same GnT-V-mediated mechanisms (56).

Coarse melanin also contained melanosomal structural protein gp100/pmel-17, and in human melanoma SKmel-23, CD63. CD63 is a lysosome/late endosome protein (70-71), and is associated with the membranes of large vesicles such as macrophage phagolysosomes, secretory granules, multivesicular bodies, and multilamellar bodies ranging in size from 0.1 to 2.4 microns (56; 72-74). In archival human melanomas, CD63 was always found associated with coarse vesicles, with or without melanin (32). As previously described (see Introduction), the association of CD63 with LAMPs 1 and 2 creates expections that this protein should co-localize to structures containing β1,6-branched N-glycans, as shown herein (56).

These studies were an outgrowth of previous work from our lab on macrophage-tumor cell fusion hybridization and metastasis (3-4, 17, 35, 37-39, 56). Fusion of leukocytes with "somatic cells" was first proposed as a mechanism of malignant transformation to explain the increased number of chromosomes in cancer cells (7-9). Spontaneous fusion of tumor cells has since been well-documented in animal tumor models (3-4). In non-cancer systems, fusion involving bone marrow-derived stem cells was an underlying basis of liver regeneration in mouse models, and has been proposed as an alternative to the concept of stem cell transdifferentiation as a mechanism for

development and maintenance of a variety of differentiated cell types (75-77). Indeed, there is considerable literature on cell-cell fusion within a variety of biological systems.

Our previous findings that artificial macrophage-melanoma fusion hybrids demonstrated elevated expression of β1,6-branched N-glycans as well as production of coarse vesicles led to the co-localization studies on cultured cells described herein, as well as to a recent survey of archival human specimens where this phenotype was observed in 22 different cancers including melanoma (32). In this same study, the coarse vesicular phenotype was particularly expressed in metastases compared with primary melanomas ($P< 0.008$), suggesting that LPHA positivity (and therefore GnT-V activity) in primary tumors may be a sign of increased metastatic competence (32).

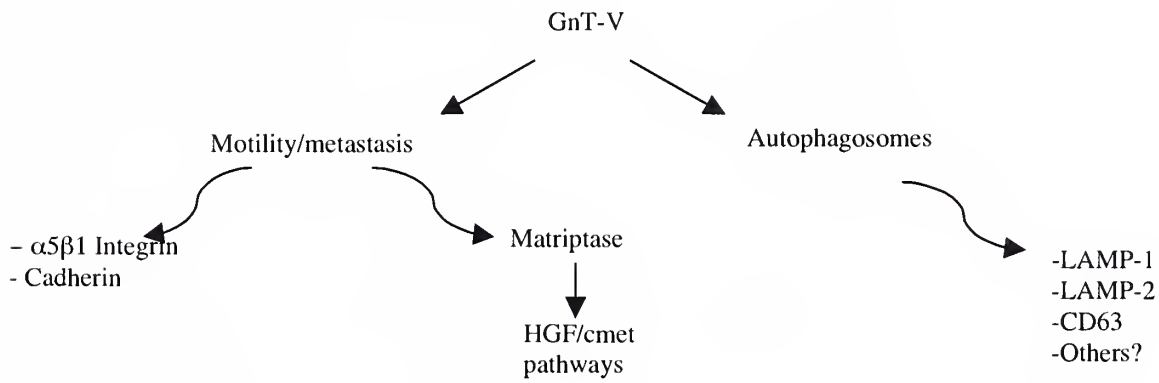
Although there are numerous regulatory systems involved in metastasis, there is a significant body of evidence that elevated GnT-V activity is an important factor. It has been shown that rodent cells transformed with Rous sarcoma/polyoma viruses or transfected with H-*ras* oncogenes have displayed increased β1,6-GlcNAc branched complex oligosaccharides and higher polylactosamine levels (78), and in another study, the loss of GnT-V activity in a glycosylation mutant of a highly metastatic cell line was associated with a loss of metastatic potential in mice (79). Very recent data also shows that the *her-2/neu* oncogene actually stimulates the transcription of GnT-V in human breast carcinoma cells via the *ras-raf-Ets* signaling pathway (80). Thus, it is reasonable to conclude that increased GnT-V activity correlates with high metastatic potential in solid tumors, but the question remains as to how (and in what genetic context) this up-regulation occurs.

Yet, despite the known association between GnT-V activity and metastatic potential, the LPHA-positive vesicular phenotype has also been inducible or observed in non-malignant cells such as the mink type II lung cells previously described as well as leiomyomas and some pigmented cutaneous nevi (81). The question thus arises as to how one might reconcile these data; a possible explanation might be that expression of the LPHA-positive phenotype in itself is insufficient to induce a metastatic transformation, and the genetic context of this expression might play a significant role in cellular behavior. In other words, GnT-V activity in fully-deregulated neoplastic (but not merely hyperplastic) cells could affect tumor progression (32).

Recent experiments in our lab involving the transfection of GnT-V (versus a "naked" plasmid control) into 6^{neo} cells revealed provocative results: In both the plasmid control as well as the GnT-V transfected cell lines, we saw an increase in pigmentation as well as the presence of coarse vesicular structures. This begged the question as to whether it was the addition of the exogenous GnT-V gene which up-regulated cellular glycosylation machinery, or whether it was simply the exposure to any foreign DNA (as in the naked plasmid) which induced cells to up-regulate native GnT-V activity. (Insofar as all cells contain the same genetic material, there must also be some baseline low level of GnT-V activity even in our control 6^{neo} cells. The point enforced thus far in this thesis is simply that the level of activity is significantly *higher* in cells of myeloid lineage.) These experiments are in the preliminary stages, however, and clearly warrant further study.

Another recent study of interest by Taniguchi et al (one of the pioneers of GnT-V research) is that addition of β1,6 GlcNAc-branching to Asn 772 in the serine protease

domain of matriptase played a pivotal role in its stability and resistance against trypsin (82). Matriptase plays important roles in cell migration, extracellular matrix degradation, and the activation of single chain urokinase-plasminogen activator and hepatocyte growth factor, all of which are involved in metastasis (83). Once activated by GnT-V induced glycosylation, matriptase cleaves pro-HGF into HGF, which acts as a ligand to cell-surface cmet receptors (with an ensuing known pathway of increased motility and metastatic potential) (83). Hence, along with glycosylation of integrins and adhesion molecules, matriptase glycosylation may constitute yet another pathway for GnT-V –induced metastatic behavior in cancer cells. The diagram below summarizes some of the potential contributions of GnT-V to metastasis thus far postulated at the molecular level:



In summary, artificial mouse macrophage-melanoma fusion hybrids demonstrated a high level of GnT-V activity consistent with previous studies, as evidenced by increased immunoperoxidase staining for LPHA (both number of cells and per-cell intensity) compared to controls. The human melanoma cells even surpassed thefusion hybrids in terms of LPHA staining positivity. Additionally, the phenotypic finding of coarse melanin previously identified by our lab in a variety of solid tumors was here confirmed in an *in vitro* model. These autophagosomes were noted to contain numerous

melanosomes, and also stained positively for LPHA, HMB45/pmel17, as well as CD63 in the human cell line. Yet, the exact significance of the coarse melanin phenotype remains elusive, and at this point we can only make note of its expression and apparent link to GnT-V enzymatic activity.

The precise molecular genetic mechanisms regulating GnT-V expression in the biological systems here described will require considerable further study, and must also be analyzed within the greater multimodal context of cancer biology. Overall, however, hybridization between melanoma cells and tumor-infiltrating macrophages appears to present a simple and unifying explanation for metastasis that is compatible with its defining characteristics of phenotypic diversity, aneuploidy, enhanced motility and aberrant glycosylation.

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